Production of Fusion Proteins

The components of the fusion protein can be linked to each other, preferably via a linker sequence. The linker sequence should separate the first and second members of the fusion protein by a distance sufficient to ensure that each member properly folds into its secondary and tertiary structures. Preferred linker sequences (1) should adopt a flexible extended conformation, (2) should not exhibit a propensity for developing an ordered secondary structure which could interact with the functional first and second component, and (3) should have minimal hydrophobic or charged character, which could promote interaction with the functional protein domains. Typical surface amino acids in flexible protein regions include Gly, Asn and Ser. Permutations of amino acid sequences containing Gly, Asn and Ser would be expected to satisfy the above criteria for a linker sequence. Other near neutral amino acids, such as Thr and Ala, can also be used in the linker sequence.

A linker sequence length of 20 amino acids can be used to provide a suitable separation of functional protein domains, although longer or shorter linker sequences may also be used. The length of the linker sequence separating the first and second components can be from 5 to 500 amino acids in length, or more preferably from 5 to 100 amino acids in length. Preferably, the linker sequence is from about 5-30 amino acids in length. In preferred embodiments, the linker sequence is from about 5 to about 20 amino acids, and is advantageously from about 10 to about 20 amino acids. Amino acid sequences useful as linkers of the first and second member include, but are not limited to, $(SerGly_4)_y$ wherein y is greater than or equal to 8, or $Gly_4SerGly_5Ser$. A preferred linker sequence has the formula $(SerGly_4)_4$. Another preferred linker has the sequence ((Ser-Ser-Ser-Ser-Gly)$_3$-Ser-Pro).

The first and second components can be directly fused without a linker sequence. Linker sequences are unnecessary where the proteins being fused have non-essential N-or C-terminal amino acid regions which can be used to separate the functional domains and

1 cycle	94°	60 sec
5 cycles	94°C	30 sec
	58°C	45 sec
	74°C	45 sec
30 cycles	94°C	30 sec
	55°C	30 sec
	74°C	30 sec

Primer sets:

GBC 332 and GBC 386, amplicon is 206 bp

GBC 332: TGTGCTCCTCTCCATGCTGG (SEQ ID NO:__)

GBC 386 TGGTCTGGGGTGACACATGT (SEQ ID NO:__)

Southern blot analysis of transgenic founders:

Genomic DNA ((24 µg total, 8 µg/lane) from each founder mouse positive for the insulator PCR was digested to completion with the restriction enzyme EcoRI. Digested DNAs were electrophoresed in triplicate and transferred to nylon membranes according to standard methods (Maniatis et al., 1982). Probes specific for each expression cassette were isolated from the VK (LC10 in pSP72, 72 bp probe), ProL (pMF141-4 in pSP72 345, bp probe), and fd-CPB (pMF213-20 in pSP72, 1861 bp probe) plasmids (provided by Michael

Table 4: Summary of ELISA and activity assays performed on the milk of mice expressing a humanized antibody fragment - enzyme fusion protein (Fab')2-CPB). (NA, not applicable)

Founder (sex, Transgenes)	F1 (transgenes)	ELISA levels (mg/ml)	Enzyme assay (mg/ml)
5 (F, LC-213)	217, 219 (LC-213)	0.092 low	0.025 low
25 (F, LC-213-141)	204 (LC-213-141)	1.5* 1.5 - 2	1.2* 1.5 - 2
67 (M, LC-213-141)	177 (LC-213-141)	NA Negative	NA negative
76 (F, LC-213)	212 (LC-213)	negative negative	negative negative
89 (F, LC-213-141)	178, 179 (LC-213-141)	1.5 - 2 1.5 - 2	1.5 -2 1.5 - 2
106 (M, LC-213-141)	186 (LC-213-141)	NA 4 - 6	NA 4 - 6
128 (F, LC-213)		low	low
152 (M, LC-213-141)	195 (LC-213-141)	NA 4 - 6	NA 4 - 6
166 (F, LC-213-141)	200, 201 (LC-213-141)	negative negative	negative negative

*Assays performed on milk collected on the second lactation of the 25 consistently gave higher values

Constructs linking the Goat Beta Casein regulatory sequences to coding region of the light and heavy chains of humanized anti-CEA F(ab')2, 806.077 fused to a modified human carboxypeptidase B enzyme, and to the coding region of the pro-domain of CPB (with C-terminal leucine) were generated. Transgenic mouse lines were generated with and without the transgene expressing the CPB pro-domain. It was demonstrated that mice transgenic for all 3 constructs are capable of producing the (Fab')2-CPB fusion at high levels (up to 4 - 6 mg/ml) in the milk of transgenic mice (4/6 triple transgenic lines expressed at levels superior to 1 mg/ml), with expected enzymatic activity. However, the absence of CPB pro-domain expression seems to correlate with low level secretion of the

active fusion protein. However this result has to be considered with caution since only 3 double transgenic lines were analyzed (only 2 both founder and F1).

In summary, variants of human pancreatic carboxypeptidase B (HCPB), with specificity for hydrolysis of C-terminal glutamic acid and aspartic acid, were prepared by site-directed mutagenesis of the human gene and expressed in the periplasm of Escherichia coli. By changing residues in the lining of the S1' pocket of the enzyme, it was possible to reverse the substrate specificity to give variants able to hydrolyse prior to C-terminal acidic amino acid residues instead of the normal C-terminal basic residues. This was achieved by mutating Asp253 at the base of the S1' specificity pocket, which normally interacts with the basic side-chain of the substrate, to either Lys or Arg. The resulting enzymes had the desired reversed polarity and enzyme activity was improved significantly with further mutations at residue 251. The [G251T,D253K]HCPB double mutant was 100 times more active against hippuryl-L-glutamic acid (hipp-Glu) as substrate than was the single mutant. [D253K]JCPB, Triple mutants, containing additional changes at Ala248, had improved activity against hipp-Glu subtrate when position 251 was Asn. These reversed polarity mutants of a human enzyme have the potential to be used in antibody-directed enzyme prodrug therapy of cancer.

EXAMPLE 2: Generation and Testing of Anti-Transferrin Receptor Antibody/ Angiogenin Fusion Constructs

This Examples shows expression of anti-transferrin receptor antibody/angiogenin fusion proteins in the mammary gland of transgenic mice. A chimeric mouse/human antibody directed against the human transferrin receptor (E6) was fused as its CH2 domain to the gene for a human angiogenic ribonuclease, angiogenin (Ang). It was expressed in the mammary gland of mice and secreted into mouse milk. Expression levels in milk were approximately 0.8 g/L. The chimeric protein retained antibody binding activity and protein synthesis inhibitory activity equivalent to that of free Ang. It was specifically cytotoxic to human tumor cells in vitro.

Materials and methods

DNA encoding the entire heavy chain of the E6 antibody, a chimeric antibody against the human transferrin receptor (Hoogenboom et al., 1990) was used between exons 2 and 7 of a modified goat ß-casein gene (Fig. 2A, I) that is expressed at high levels in the milk of lactating transgenic mice (Roberts et al., 1992). A second transgene encoding an antibody-enzyme fusion was prepared by linking the gene for the human RNase, angiogenin (Ang) to the CH2 domain of the antibody (Fig. 1 and Fig. 2A, II). Those genes as well as the gene encoding the light chain of the same antibody (Fig. 2A, III) were all cloned separately, and the appropriate pairs (heavy (H) and light (L) chains; CH2Ang and L chain) were purified free of procaryotic DNA and co-injected into mouse embryos that were reimplanted using standard methods (Roberts et al., 1992). Transgenic mice were identified by PCR and southern blot analysis of DNA obtained from tails of the resulting progeny.

Founder mice were bred to produce lactating transgenic females. Milk was collected, diluted with PBS and analyzed for the presence of the antibody chains and Ang. Polyclonal antibodies raised against human Ang only reacted with a band of the expected M (43 kDa; antibody heavy chain, 29 kDA; Ang, 14 kDA) in the fusion protein (Fig. 2B, left panel). However, anti-IgG antisera strongly reacted with both the H and L chains of the chimeric E6 antibody (Fig. 2B, right panel). Whereas the L chain of the antibody fusion protein was clearly observed with the anti-IgG antisera, the truncated H chain of CH2Ang was barely detectable suggesting that the fusion of angiogenin to the CH2 domain hindered binding of the antisera to the H chain.

The chimeric IgG antibody was purified by chromatography on Protein A Sepharose. As shown in Fig. 2C, lanes 1 and 2, Western analysis of the final purified product by gel electrophoresis under reducing conditions showed the presence of light (28 kDa) and heavy chain proteins (approximately 55 kDa). Western analysis under non-reducing conditions (Fig. 2C, lane 3) demonstrated that the transgenic antibody existed as a mixture of IgG and Fab forms (168 and 84 kDa, respectively). A small amount of free heavy chain (55 kDa) was also seen.

Milk containing the CH2Ang fusion protein was similarly collected and diluted with PBS. Protein A Sepharose failed to bind the angiogenin fusion protein. Analogous results were obtained when the same CH2 antibody fragment previously was fused to TNF and it